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Oxidation-Specific Biomarkers and Risk of Peripheral Artery Disease

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Objectives The goal of this study was to examine the prospective association between oxidation-specific biomarkers, primarily oxidized phospholipids (OxPL) on apolipoprotein B-100–containing lipoproteins (OxPL/apoB) and lipoprotein (a) [Lp(a)], and risk of peripheral artery disease (PAD). We examined, as secondary analyses, indirect measures of oxidized lipoproteins, including autoantibodies to malondialdehyde-modified low-density lipoprotein (MDA-LDL) and apolipoprotein B-100 immune complexes (ApoB-IC).

Background Biomarkers to predict the development of PAD are lacking. OxPL circulate in plasma, are transported by Lp(a), and deposit in the vascular wall and induce local inflammation.

Methods The study population included 2 parallel nested case-control studies of 143 men within the Health Professionals Follow-up Study (1994 to 2008) and 144 women within the Nurses' Health Study (1990 to 2010) with incident confirmed cases of clinically significant PAD, matched 1:3 to control subjects.

Results Levels of OxPL/apoB were positively associated with risk of PAD in men and women: pooled relative risk: 1.37, 95% confidence interval: 1.19 to 1.58 for each 1-SD increase after adjusting age, smoking, fasting status, month of blood draw, lipids, body mass index, and other cardiovascular disease risk factors. Lp(a) was similarly associated with risk of PAD (pooled adjusted relative risk: 1.36; 95% confidence interval: 1.18 to 1.57 for each 1-SD increase). Autoantibodies to MDA-LDL and ApoB-IC were not consistently associated with risk of PAD.

Conclusions OxPL/apoB were positively associated with risk of PAD in men and women. The major lipoprotein carrier of OxPL, Lp(a), was also associated with risk of PAD, reinforcing the key role of OxPL in the pathophysiology of atherosclerosis mediated by Lp(a). (J Am Coll Cardiol 2013;61:2169–79) © 2013 by the American College of Cardiology Foundation

Approximately 10 million U.S. adults have peripheral artery disease (PAD), including 23% of those 70 years of age or older (1,2). Peripheral artery disease is associated with substantial morbidity, cost (3), and functional decline (4) and might require limb amputation in extreme cases. Despite its high prevalence and associated morbidity, risk factors for PAD are less well-studied than those for coronary and cerebrovascular disease.

Evidence from cellular and animal experiments suggests that oxidative stress plays a key, modifiable role in the etiology of atherosclerosis (5,6). However, there is a lack of appropriate epidemiological markers to measure oxidation:

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Abbreviations and Acronyms

ABI = ankle-brachial index
ApoB = apolipoprotein B-100–containing lipoproteins
ApoB-IC = apoB-immune complexes
BMI = body mass index
BSA = bovine serum albumin
CI = confidence interval
CVD = cardiovascular disease
Ig = immunoglobulin
LDL = low-density lipoprotein
Lp(a) = lipoprotein (a)
MDA = malondialdehyde
MI = myocardial infarction
OxPL = oxidized phospholipids
PAD = peripheral artery disease
PC = phosphocholine
RLU = relative light units
RR = incidence rate ratio/ relative risk

many biomarkers lack the necessary combination of reliability, accuracy, cost-effectiveness, and ease of measurement; and few have been examined specifically with respect to PAD. Oxidized phospholipids (OxPL), a marker of lipid oxidation transported by lipoprotein (a) [Lp(a)] in plasma (7,8), might provide insight into the role of oxidative stress in atherosclerosis. Pro-inflammatory OxPL on Lp(a) and other apoB-100–containing lipoproteins upregulate proinflammatory genes and proinflammatory responses of several arterial wall cells and initiate a localized inflammatory cascade (9,10).

These oxidation-specific epitopes—such as OxPL and malondialdehyde (MDA) epitopes—represent danger-associated molecular patterns that are detrimental to the host; are present on apoptotic cells, oxidized low-density lipoprotein (LDL), and lipids; and often share molecular identity/mimicry with epitopes on pathogens (11). In response to such danger-associated molecular patterns, macrophage scavenger receptors, immune effector proteins such as autoantibodies to malondialdehyde-modified low-density lipoprotein (MDA-LDL), complement factor H (12), and C-reactive protein have been selected and expanded to bind and neutralize their pro-inflammatory effects.

Oxidized phospholipids on apolipoprotein B-100–containing lipoproteins (OxPL/apoB) have been associated with carotid and femoral atherosclerosis measured by ultrasound (13), myocardial infarction (MI), stroke, revascularization, and total mortality in selected, largely clinical populations (7). Immunoglobulin (Ig) M autoantibodies to MDA-LDL have also been inversely associated with ultrasound-detected carotid and femoral atherosclerosis (14,15); and in another small cross-sectional study, autoantibody titers against oxidized LDL were increased in patients with early onset PAD (16). However, no adequately powered prospective cohort studies have examined the association between oxidation-specific biomarkers and risk of incident clinically manifested PAD in healthy populations. The goal of this study was to examine the prospective association between oxidation-specific biomarkers, primarily oxidized phospholipids (OxPL) on apolipoprotein B-100–containing lipoproteins (OxPL/apoB) and lipoprotein (a) [Lp(a)], and risk of peripheral artery disease (PAD). We examined, as secondary analyses, indirect measures of oxidized lipoproteins, including autoantibodies to malondialdehyde-modified low-density lipoprotein (MDA-LDL) and apolipoprotein B-100 immune complexes (ApoB-IC).

Methods

Study population. The HPFS (Health Professionals Follow-up Study) is a prospective cohort study of 51,529 male dentists, optometrists, pharmacists, podiatrists, osteopathic physicians, and veterinarians 40 to 75 years of age that began in 1986. The NHS study (Nurses' Health Study) is a prospective cohort study of 121,700 female nurses 30 to 55 years of age that began in 1976. From individuals in these 2 studies, 18,224 men provided blood specimens in 1994, and 32,826 women provided a blood sample in 1989. We excluded individuals who had a history of cardiovascular disease (CVD)—including MI; surgical/percutaneous revascularization of the coronary, carotid, or peripheral beds; confirmed PAD; stroke; and transient ischemic attack.

The case-control analytic datasets include 143 incident cases and 429 control subjects in the HPFS study and 144 incident cases and 432 control subjects in the NHS study. Cases were matched 1:3 to control subjects on age, race (NHS study only), month of blood draw (within 3 months), fasting status, and smoking history (never/former/current). We selected control subjects at random, conditional on the matching factors, from participants free of PAD at the time the case occurred (risk set sampling). Because older age categories had fewer participants, we relaxed the age match range year-by-year if necessary to a maximum of within 3 years. The Harvard School of Public Health Human Subjects Committee approved both studies.

Assessment of OxPL, Lp(a), autoantibodies, and immune complexes. Blood samples were shipped overnight with a cold pack to the central laboratory, centrifuged on arrival, aliquotted, and stored in liquid nitrogen at −130°C to −196°C. The HPFS specimens were anticoagulated with ethylenediaminetetraacetic acid, and NHS specimens were anticoagulated with heparin, and 95% of HPFS bloods and 97% of NHS bloods were received within 24 h. A validated plasma assay was used to measure OxPL/apoB, with the murine monoclonal antibody E06 that recognizes the phosphocholine (PC) group on oxidized but not on native phospholipids (reviewed in detail in Taleb et al. [7] and references therein). E06 similarly recognizes the PC covalently bound to bovine serum albumin (BSA), as in PC-BSA. A 1:50 dilution of plasma in phosphate-buffered saline is added to microtiter wells coated with monoclonal antibody MB47, which binds a saturating amount of apoB-100 to each well. Finally, biotinylated E06 is used to determine OxPL/apoB in relative light units (RLU). Within-person 5-year reproducibility of frozen samples is high (r = 0.78) (13), and pilot-tests showed that OxPL/apoB levels are stable over 24 h on ice (intra-class correlation coefficient = 0.96).

To facilitate comparison of absolute OxPL/apoB levels across studies, we also describe the reporting of OxPL/apoB levels as nanomolar (nmol/l or nM) OxPL rather than RLU OxPL with a novel standard curve of PC equivalents. The standard curve is generated by plating known concentrations of phosphocholine-modified bovine serum albumin (PC-BSA), which has approximately 16 mole of PC/mole of BSA (Biotech Technologies, Novato, California), and is recognized by E06. Biotin-E06 is then added to detect the number of moles of PC in the linear range on the plate and is measured in RLU. This standard curve is then used to convert the RLU derived from wells containing individual human samples to OxPL equivalents. This new method is reported as nanomoles of PC found on OxPL/l plasma (i.e., nanomolar or nM) for each sample. Because each mole of OxPL has 1 PC headgroup recognized by E06, this can be reported as nM OxPL.

The Lp(a) levels were determined with a chemiluminescent enzyme-linked immunoadsorbent assay with MB47-coated wells, a 1:400 plasma dilution, and biotinylated monoclonal antibody LPA4, as described previously (17). Chemiluminescent enzyme-linked immunoadsorbent assay was used to measure IgG and IgM autoantibodies to MDA-LDL and apoB-immune complexes (ApoB-IC), as previously described (18). Measured coefficients of variation in duplicate samples were 8% for OxPL/apoB, 13% for Lp(a), 11% for IgG autoantibodies, 9% for IgM autoantibodies, 10% for IgG ApoB-IC, and 10% for IgM ApoB-IC in the HPFS study. Coefficients of variation were 21% for OxPL/apoB, 13% for Lp(a), 9% for IgG autoantibodies, 7% for IgM autoantibodies, 16% for IgG ApoB-IC, and 25% for IgM ApoB-IC in the NHS study.

Assessment of PAD. Participants reported the occurrence of professionally diagnosed medical conditions, including claudication and revascularization for arterial disease of the leg, during the previous 2 years on biennial mailed questionnaires. We collected medical records from treating physicians and hospitals for those who reported either condition. Professionals blinded to biomarker status reviewed and confirmed PAD diagnoses with medical records.

We defined PAD as arterial disease below the aortic bifurcation (i.e., excluding abdominal aortic aneurysm and renal artery stenosis) as described previously (19). Confirmed PAD required at least 1 of the following (in order of severity/certainty): 1) report of amputation, bypass, or other revascularization procedure (e.g., angioplasty) for occlusive artery disease; 2) angiogram showing at least 50% stenosis of at least 1 artery with congruent symptoms in the ipsilateral limb; 3) ankle-brachial index (ABI) <0.9; or 4) diagnosis of physician confirmed by medical record review.

Assessment of covariates. Questionnaires provided information about medical history and lifestyle habits, including detailed information about smoking, medication use, weight, parental history of MI, alcohol, physical activity, diet, and post-menopausal hormone use. Participants reported the average amount of time they spent/week on various activities such as walking, jogging, running, bicycling, and tennis. This information was used to calculate weekly energy expenditure in metabolic equivalent task-hours. We calculated body mass index (BMI) by dividing weight (kg) by squared height (m^2). Both physical activity and BMI measures are highly valid (20–22).

A laboratory certified by the National Heart, Lung and Blood Institute/Centers for Disease Control and Prevention Lipid Standardization Program analyzed all other biochemical markers by means of commercially available analytic systems. The laboratory measured high-density lipoprotein cholesterol and triglycerides enzymatically and LDL cholesterol by a homogenous direct method from Roche Diagnostics (Indianapolis, Indiana). An immunoturbidimetric assay on the Roche P Modular system from Roche Diagnostics quantified the concentration of high-sensitivity C-reactive protein, with reagents and calibrators from DiaSorin (Stillwater, Minnesota). The Roche P Modular system uses turbidimetric immunoinhibition and hemolyzed whole blood or packed red cells to determine hemoglobin A_{1c} (Roche Diagnostics, Indianapolis, Indiana).

Statistical analysis. We used generalized linear mixed models and Cochran-Mantel-Haenszel tests to compare continuous variables and categorical variables by case/control status, respectively, accounting for clustering by matching status. We examined the shape of the associations of OxPL/apoB, Lp(a), autoantibodies, and ApoB-IC with PAD risk with cubic splines, adjusting for all covariates. We used conditional logistic regression, conditioning on matching factors, to estimate odds ratios for PAD according to level of OxPL/apoB and present estimates adjusted for matching factors and additionally for other PAD risk factors. Because we employed risk set sampling, we report these odds ratios as unbiased estimates of the incidence rate ratio (RR).

We included covariates in our models as linear variables if appropriate or as categorical variables if discrete or their association with PAD was nonlinear. We checked for multicollinearity among covariates with variance inflation factors. Because the test for heterogeneity was not statistically significant, we used meta-analysis with fixed effects to pool our RR estimates for OxPL/apoB and Lp(a). We tested effect modification by including interaction terms between OxPL/apoB and the potential effect modifier (continuous variable) in our models and pooled the interaction terms for men and women with a meta-analysis with fixed effects. We analyzed levels of autoantibodies and ApoB-IC in similar fashion. All analyses used SAS statistical software (version 9.2, Cary, North Carolina).

Results

Among men, medical reports of amputation, bypass, or other revascularization procedure confirmed 87 PAD cases (61%), angiogram or Doppler ultrasound confirmed 15 cases

Table 1 Baseline Characteristics of Cases and Matched Control Subjects

	Women			Men		
	Cases (n = 144)	Control Subjects (n = 432)	p Value*	Cases (n = 143)	Control Subjects (n = 429)	p Value*
Age (yrs)	59.9 ± 5.2	60.0 ± 5.2	Matched	65.4 ± 8.1	65.3 ± 8.1	Matched
Oxidation-related factors						
OxPL/apoB (nM)	8.46 (3.9–19.3)	5.00 (2.9–10.6)	<0.001	15.24 (12.4–23.6)	14.15 (12.3–19.4)	0.02
OxPL/apoB (RLU)	4,882 (2,237–11,135)	2,887 (1,649–6,132)	<0.001	8,800 (7,162–1,3601)	8,173 (7,126–11,184)	0.02
Lipoprotein (a) (mg/dl)	35.1 (8.7–72.3)	13.1 (4.9–43.1)	<0.001	7.6 (2.8–34.3)	4.5 (1.6–17.7)	0.007
IgG AA to MDA-LDL (RLU)	8,776 (6,394–12,669)	8,541 (5,572–12,758)	0.34	2,313 (1,411–4,949)	2,733 (1,741–4,350)	0.36
IgM AA to MDA-LDL (RLU)	20,457 (14,001–26,529)	18,866 (13,903–25,145)	0.22	9,684 (5,836–14,986)	9,213 (6,363–13,823)	0.16
IgG ApoB-IC (RLU)	1,106 (862–1,439)	1,076 (791–1,494)	0.94	1,620 (1,265–2,243)	1748 (1,376–2,374)	0.22
IgM ApoB-IC (RLU)	1,490 (897–2,574)	1,478 (960–2,393)	0.17	885 (562–1,600)	921 (601–1,425)	0.29
Lipids						
Triglycerides (mg/dl)	110 (85–161)	106 (73–145)	0.34	143 (105–195)	115 (80–165)	0.001
HDL-C (mg/dl)	60.5 ± 19.9	62.1 ± 17.1	0.42	41.7 ± 11	48.5 ± 14	<0.001
LDL-C (mg/dl)	148 ± 44	142 ± 38	0.16	139 ± 35	131 ± 33	0.02
hsCRP (mg/l)	2.56 (1.25–4.70)	1.62 (0.73–3.33)	0.07	2.24 (1.2–3.5)	1.18 (0.5–2.3)	0.005
HbA_{1c} (%)	5.46 (5.28–5.72)	5.33 (5.16–5.55)	<0.001	5.56 (5.3–6.0)	5.41 (5.2–5.6)	<0.001
Smoking status						
Never	30 (21%)	90 (21%)	Matched	23 (18%)	82 (20%)	Matched
Past	56 (39%)	170 (39%)	Matched	78 (60%)	242 (59%)	Matched
Current	58 (40%)	172 (40%)	Matched	32 (22%)	90 (21%)	Matched
1–14 cigarettes/day	18 (13%)	86 (20%)		15 (11%)	44 (11%)	
15–34 cigarettes/day	31 (22%)	80 (19%)		11 (8%)	33 (8%)	
35+ cigarettes/day	9 (6%)	5 (1%)		4 (3%)	9 (2%)	
Pack-yrs (yrs)	32.3 ± 25.6	22.0 ± 21.3	<0.001	28.7 ± 24	22.5 ± 22	<0.001
Physical activity (MET h/wk)	12.8 (4.7–24.9)	13.6 (5.0–28.1)	0.69	22.7 (8.0–43.8)	27.4 (10.3–52.8)	0.003
History of hypertension	68 (47%)	137 (32%)	<0.001	70 (49%)	130 (30%)	<0.001
History of diabetes	19 (13%)	12 (3%)	<0.001	28 (20%)	16 (4%)	<0.001
History of hypercholesterolemia	84 (58%)	200 (46%)	0.01	82 (57%)	187 (44%)	0.005
Alcohol						
Never drinker	49 (34%)	130 (30%)		35 (24%)	84 (20%)	
Former drinker	44 (31%)	137 (32%)		24 (17%)	80 (19%)	
<1 drink/day	29 (20%)	109 (25%)	0.43	39 (27%)	123 (29%)	0.81
1–1.9 drinks/day	12 (8%)	20 (5%)		23 (16%)	65 (15%)	
2+ drinks/day	9 (6%)	31 (7%)		22 (15%)	76 (18%)	
Parental history of MI <age 60 yrs	31 (22%)	61 (14%)	0.03	22 (15%)	44 (10%)	0.09
BMI category						
<25	84 (58%)	259 (60%)		62 (43%)	204 (48%)	
25–29.9	38 (26%)	132 (31%)	0.13	67 (47%)	196 (46%)	0.42
30+	22 (15%)	41 (9%)		14 (10%)	29 (7%)	
Aspirin use	29 (20%)	89 (21%)	0.91	80 (56%)	184 (43%)	0.01
Post-menopausal	131 (95%)	390 (95%)	0.72			
Ever used post-menopausal hormones†	96 (72%)	257 (63%)	0.04			
Currently using post-menopausal hormones	57 (43%)	183 (45%)	0.84			

Values are mean ± SD, median (interquartile range), or n (%). *Generalized linear mixed models for continuous variables and Cochran-Mantel-Haenszel test for categorical variables (to account for matching/correlation between control subjects); matching criteria were age, month of blood draw, fasting status, and smoking status. †Among post-menopausal women.

AA to MDA-LDL = autoantibodies to malondialdehyde-modified low-density lipoprotein cholesterol; ApoB-IC = apolipoprotein B-100 immune complexes; BMI = body mass index; hsCRP = high-sensitivity C-reactive protein; HbA_{1c} = hemoglobin A_{1c}; HDL-C = high-density lipoprotein cholesterol; Ig = immunoglobulin; LDL-C = low-density lipoprotein cholesterol; MET h = metabolic equivalent task-hours; MI = myocardial infarction; OxPL/apoB = oxidized phospholipids on apolipoprotein B-100–containing lipoproteins; RLU = relative light units.

(10%), ABI <0.9 confirmed 23 cases (16%), and physician diagnosis confirmed 18 cases (13%). Among women, surgery or procedure confirmed 74 cases (51%), angiogram confirmed 24 cases (17%), ABI confirmed 39 cases (27%), and physician diagnosis confirmed 7 cases (5%). Compared with control subjects, PAD cases had higher levels of traditional CVD risk factors (Table 1). Although cases and control subjects were matched on current smoking status, cases still had a significantly higher average number of pack-years compared with control subjects.

OxPL/apoB and Lp(a). Women had considerably lower levels of OxPL/apoB compared with men, whereas the distribution of Lp(a) overlapped substantially across sexes, with somewhat greater levels among women. OxPL/apoB

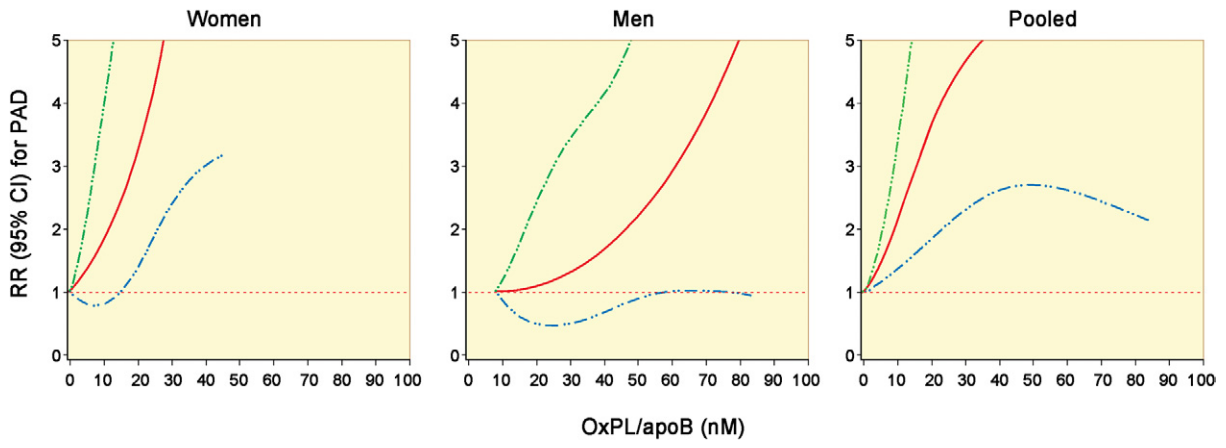

Figure 1 **Adjusted Relationship Between Plasma Levels of OxPL/apoB and RR of PAD**

Data derive from a cubic spline conditional logistic regression model with age, race (women only), smoking status, fasting status, and date of blood sampling as matching variables. The model is adjusted for triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, high-sensitivity C-reactive protein, hemoglobin A_{1c}, history of diabetes, history of hypertension, pack-years of smoking, parental history of myocardial infarction before age 60, aspirin use, body mass index, physical activity, post-menopausal hormone use (women only), and sex (pooled only). The 95% confidence interval (CI) is indicated by the dashed lines. ApoB = apolipoprotein B-100–containing lipoproteins; OxPL = oxidized phospholipids; PAD = peripheral artery disease; RR = relative risk.

was not strongly correlated with standard CVD risk factors (Online Table 1).

The association between OxPL/apoB and Lp(a) and risk of PAD appeared linear (Figs. 1 to 3), and likelihood ratio tests for nonlinearity were not statistically significant ($p > 0.05$), comparing a model with the linear term with a model with the linear and cubic spline terms. Therefore we discuss our results with units of 1 SD of nM OxPL/apoB. We have additionally presented our results with RLU, because all previous work used OxPL/apoB measured in RLU. There was a 51% (95% confidence interval [CI]: 24% to 85%) increase in risk of PAD for each 1-SD increase in OxPL/apoB in women and a 23% (95% CI: 0% to 52%) increase in men in full multivariable models (Table 2). These estimates were essentially the same for matching-adjusted models (53% in women, and 24% in men). Similarly, there was a 52% (95% CI: 24% to 87%) increase in risk of PAD for each 1-SD increase in Lp(a) in women, and a 24% (95% CI: 0% to 53%) increase in men in full multivariable models (Table 3).

Because we did not observe statistically significant heterogeneity between the NHS and HPFS studies, we pooled our results for OxPL/apoB and Lp(a) in women and men (Tables 2 and 3). In pooled analyses, a 1-SD increase in OxPL/apoB was associated with a 37% (95% CI: 19% to 58%) increased risk of PAD, adjusting for all covariates. A

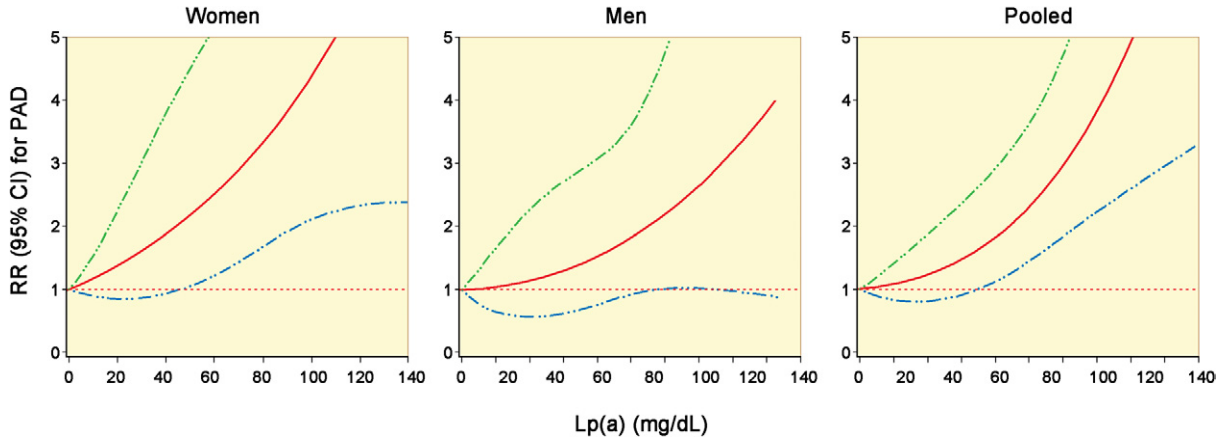

Figure 2 **Adjusted Relationship Between Plasma Levels of Lp(a) and RR of PAD**

Lp(a) = lipoprotein (a); other abbreviations as in Figure 1.

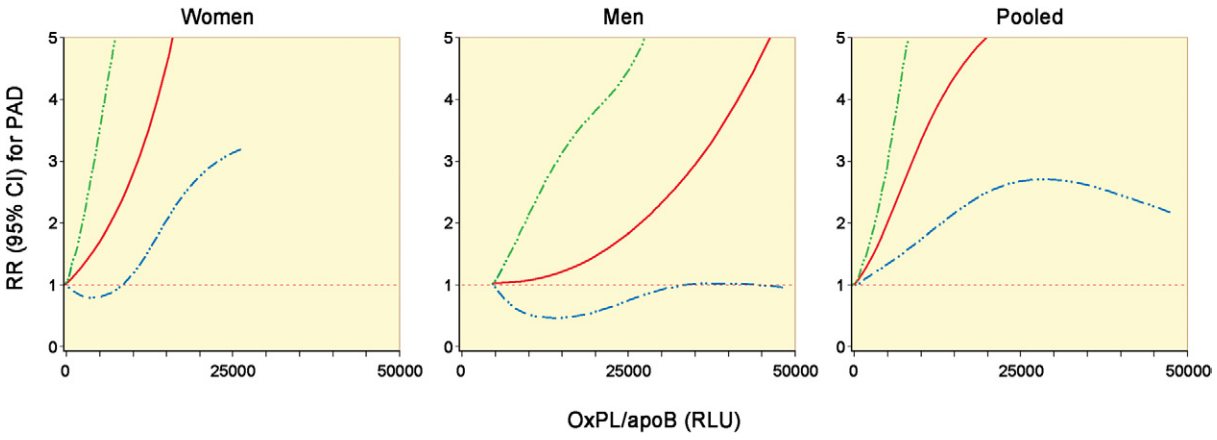

Figure 3 Adjusted Relationship Between Plasma Levels of OxPL/apoB (RLU) and RR of PAD

RLU = relative light units; other abbreviations as in Figure 1.

1-SD increase in Lp(a) was associated with almost the same magnitude of increased risk of PAD. With tertiles to categorize OxPL/apoB and Lp(a), men and women in the highest compared with lowest tertile of both biomarkers had approximately double the risk of PAD. In additional analyses, we found no interactions of OxPL/apoB with age, years of follow-up, LDL cholesterol, autoantibodies to MDA-LDL, or ApoB-IC.

IgG and IgM autoantibodies and immune complexes. We did not find consistent associations of autoantibodies to MDA-LDL with risk of PAD. The relative risks fluctuated across tertiles and the CIs were consistent with a broad range of risk estimates (Table 4), although it was of interest that there was a significant inverse relationship of IgG to MDA-LDL in men but not women. The IgG and IgM ApoB-IC were not statistically significantly associated with

Table 2 Relative Risks and 95% CIs for Peripheral Artery Disease According to Level of OxPL/ApoB

Women									
	Per 1-SD Increase (7.17)	**Tertile**				**Per 1-SD Increase (4,142)**	**Tertile**		
		1	**2**	**3**			**1**	**2**	**3**
Range (nM)		0.00–3.72	3.73–9.55	9.56–46.09	Range (RLU)		0–2,150	2,151–5,517	5,518–26,622
Cases	144	33	43	68	Cases	144	33	43	68
Model 1	1.53 (1.30–1.79)	1.0 (ref)	1.44 (0.86–2.41)	2.73 (1.67–4.48)	Model 1	1.53 (1.30–1.79)	1.0 (ref)	1.44 (0.86–2.41)	2.73 (1.67–4.48)
Model 2	1.50 (1.26–1.79)	1.0 (ref)	1.58 (0.91–2.75)	2.92 (1.71–5.00)	Model 2	1.50 (1.26–1.79)	1.0 (ref)	1.58 (0.91–2.75)	2.92 (1.71–5.00)
Model 3	1.51 (1.24–1.85)	1.0 (ref)	1.38 (0.75–2.54)	2.55 (1.41–4.64)	Model 3	1.51 (1.24–1.85)	1.0 (ref)	1.38 (0.75–2.54)	2.55 (1.41–4.64)
Men									
	Per 1-SD Increase (10.38)	**Tertile**				**Per 1-SD Increase (5,999)**	**Tertile**		
		1	**2**	**3**			**1**	**2**	**3**
Range (nM)		7.85–12.93	12.94–16.85	16.86–82.75	Range (RLU)		4,535–7,518	7,519–9,730	9,731–47,792
Cases	143	41	47	55	Cases	143	42	46	55
Model 1	1.24 (1.05–1.46)	1.0 (ref)	1.15 (0.70–1.87)	1.50 (0.92–2.46)	Model 1	1.24 (1.05–1.46)	1.0 (ref)	1.09 (0.67–1.78)	1.46 (0.90–2.38)
Model 2	1.31 (1.09–1.58)	1.0 (ref)	1.23 (0.72–2.08)	1.78 (1.03–3.09)	Model 2	1.31 (1.09–1.58)	1.0 (ref)	1.15 (0.68–1.96)	1.72 (0.99–2.97)
Model 3	1.23 (1.00–1.52)	1.0 (ref)	1.03 (0.57–1.86)	1.50 (0.81–2.78)	Model 3	1.23 (1.00–1.52)	1.0 (ref)	0.99 (0.55–1.79)	1.46 (0.79–2.70)
Pooled									
Model 3	1.37 (1.19–1.58)	1.0 (ref)	1.18 (0.77–1.82)	1.97 (1.28–3.03)	Model 3	1.37 (1.18–1.58)	1.0 (ref)	1.17 (0.76–1.80)	1.95 (1.27–3.00)

Values are relative risk (95% CI) unless otherwise indicated. Model 1: adjusted for matching factors (age, race [women only], month of blood draw, fasting status, and smoking). Model 2: adjusted for matching factors, triglycerides, HDL-C, LDL-C, hsCRP, and HbA_{1c}. Model 3: adjusted for matching factors, triglycerides, HDL-C, LDL-C, hsCRP, HbA_{1c}, parental history of MI before 60 years of age, pack-years of smoking, physical activity, hypertension, diabetes, hypercholesterolemia, BMI, aspirin use, and post-menopausal hormone use (women only).

CI = confidence interval; other abbreviations as in Table 1.

Table 3 Relative Risks and 95% CIs for Peripheral Artery Disease According to Level of Lp(a)

	Per 1-SD Increase (28.8)	Tertile 1	Tertile 2	Tertile 3
Women				
Range (mg/dl)		0.1–8.4	8.5–35.0	35.1–144.6
Cases	144	35	37	72
Model 1	1.57 (1.33–1.86)	1.0 (ref)	1.04 (0.62–1.75)	2.64 (1.64–4.26)
Model 2	1.54 (1.28–1.85)	1.0 (ref)	1.11 (0.64–1.94)	2.76 (1.64–4.67)
Model 3	1.52 (1.24–1.87)	1.0 (ref)	1.37 (0.72–2.59)	2.91 (1.58–5.36)
Men				
	Per 1-SD Increase (22.8)	Tertile 1	Tertile 2	Tertile 3
Range (mg/dl)		0.1–2.4	2.5–12.0	12.1–129.1
Cases	143	31	52	60
Model 1	1.29 (1.09–1.52)	1.0 (ref)	1.88 (1.13–3.13)	2.51 (1.49–4.21)
Model 2	1.27 (1.05–1.53)	1.0 (ref)	1.27 (0.73–2.22)	1.91 (1.08–3.39)
Model 3	1.24 (1.00–1.53)	1.0 (ref)	1.20 (0.64–2.25)	1.59 (0.84–3.00)
Pooled				
Model 3	1.36 (1.18–1.57)	1.0 (ref)	1.28 (0.82–2.00)	2.18 (1.40–3.39)

Values are relative risk (95% CI) unless otherwise indicated. Model 1: adjusted for matching factors (age, race [women only], month of blood draw, fasting status, and smoking). Model 2: adjusted for matching factors, triglycerides, HDL-C, LDL-C, hsCRP, and HbA_{1c}. Model 3: adjusted for matching factors, triglycerides, HDL-C, LDL-C, hsCRP, HbA_{1c}, parental history of MI before 60 years of age, pack-years of smoking, physical activity, hypertension, diabetes, hypercholesterolemia, BMI, aspirin use, and post-menopausal hormone use (women only).

Lp(a) = lipoprotein (a); other abbreviations as in Table 1.

risk of PAD in men or in women, either as continuous variables or in tertiles (Table 5).

OxPL/apoB and Lp(a) in context. In women, the C-statistic value for area under the receiver-operating characteristic curve increased from 0.725 to 0.756 and 0.761 with the addition of OxPL/apoB and Lp(a), respectively, to a list of traditional risk factors (Table 6). These risk factors include age, race (women only), month of blood draw, fasting status, smoking status, parental history of MI before age 60, pack-years of smoking,

Table 4 Relative Risks and 95% CIs for Peripheral Artery Disease According to Level of AA to MDA-LDL

	IgG AA				IgM AA			
Women								
	Per 1-SD Increase log(IgG AA)	Tertile 1	Tertile 2	Tertile 3	Per 1-SD Increase	Tertile 1	Tertile 2	Tertile 3
Range (RLU)		1,465–6,580	6,581–11,017	11,018–57,009		2,013–15,741	15,742–22,494	22,495–77,968
Cases	144	40	56	48	144	50	34	60
Model 1	1.16 (0.96–1.40)	1.0 (ref)	1.53 (0.95–2.45)	1.26 (0.78–2.02)	1.13 (0.95–1.36)	1.0 (ref)	0.59 (0.35–0.98)	1.34 (0.84–2.13)
Model 2	1.16 (0.95–1.42)	1.0 (ref)	1.48 (0.89–2.46)	1.32 (0.80–2.17)	1.10 (0.91–1.34)	1.0 (ref)	0.57 (0.34–0.97)	1.32 (0.81–2.17)
Model 3	1.10 (0.88–1.37)	1.0 (ref)	1.42 (0.79–2.53)	1.13 (0.65–1.96)	1.00 (0.80–1.26)	1.0 (ref)	0.47 (0.26–0.85)	1.05 (0.60–1.85)
Men								
	Per 1-SD Increase log(IgG AA)	Tertile 1	Tertile 2	Tertile 3	Per 1-SD Increase	Tertile 1	Tertile 2	Tertile 3
Range (RLU)		485–1,940	1,941–3,690	3,691–64,956		732–7,203	7,204–11,833	11,834–94,623
Cases	143	57	40	46	143	45	44	54
Model 1	0.94 (0.79–1.12)	1.0 (ref)	0.57 (0.35–0.93)	0.73 (0.46–1.15)	1.13 (0.96–1.34)	1.0 (ref)	0.91 (0.56–1.49)	1.25 (0.80–1.97)
Model 2	0.87 (0.71–1.06)	1.0 (ref)	0.52 (0.30–0.89)	0.54 (0.32–0.92)	1.18 (0.97–1.43)	1.0 (ref)	0.83 (0.48–1.43)	1.17 (0.70–1.96)
Model 3	0.84 (0.67–1.06)	1.0 (ref)	0.58 (0.31–1.08)	0.52 (0.29–0.95)	1.13 (0.93–1.39)	1.0 (ref)	1.02 (0.55–1.87)	1.26 (0.71–2.23)

Values are relative risk (95% CI) unless otherwise indicated. Model 1: adjusted for matching factors (age, race [women only], month of blood draw, fasting status, and smoking status). Model 2: adjusted for matching factors, triglycerides, HDL-C, LDL-C, hsCRP, and HbA_{1c}. Model 3: adjusted for matching factors, triglycerides, HDL-C, LDL-C, hsCRP, HbA_{1c}, parental history of MI before 60 years of age, pack-years of smoking, physical activity, hypertension, diabetes, hypercholesterolemia, BMI, aspirin use, and post-menopausal hormone use (women only).

Abbreviations as in Table 1.

Table 5 Relative Risks and 95% CIs for Peripheral Artery Disease According to Level of IC

	IgG ApoB-IC				IgM ApoB-IC			
Women								
	Per 1-SD Increase	Tertile 1	Tertile 2	Tertile 3	Per 1-SD Increase	Tertile 1	Tertile 2	Tertile 3
Range (RLU)		272–906	907–1,328	1,329–12,372		23–1,142	1,143–2,050	2,051–21,500
Cases	144	41	58	45	144	49	52	53
Model 1	1.00 (0.82–1.21)	1.0 (ref)	1.53 (0.96–2.44)	1.15 (0.71–1.88)	1.13 (0.95–1.33)	1.0 (ref)	0.78 (0.48–1.26)	1.13 (0.71–1.80)
Model 2	0.97 (0.78–1.20)	1.0 (ref)	1.55 (0.94–2.55)	1.08 (0.64–1.83)	1.15 (0.96–1.38)	1.0 (ref)	0.82 (0.49–1.38)	1.21 (0.74–1.98)
Model 3	0.96 (0.73–1.24)	1.0 (ref)	1.67 (0.96–2.93)	0.94 (0.51–1.74)	1.14 (0.92–1.41)	1.0 (ref)	0.78 (0.45–1.37)	1.08 (0.61–1.91)
Men								
	Per 1-SD Increase	Tertile 1	Tertile 2	Tertile 3	Per 1-SD Increase	Tertile 1	Tertile 2	Tertile 3
Range (RLU)		459–1,460	1,461–2,061	2,062–28,725		96–685	686–1,235	1,236–59,411
Cases	143	54	43	46	143	49	47	47
Model 1	0.88 (0.68–1.13)	1.0 (ref)	0.68 (0.43–1.08)	0.77 (0.48–1.24)	1.08 (0.97–1.21)	1.0 (ref)	0.90 (0.57–1.43)	0.94 (0.60–1.48)
Model 2	0.89 (0.68–1.15)	1.0 (ref)	0.65 (0.38–1.09)	0.84 (0.49–1.44)	1.10 (0.93–1.31)	1.0 (ref)	0.90 (0.54–1.52)	0.97 (0.59–1.59)
Model 3	0.86 (0.63–1.17)	1.0 (ref)	0.65 (0.36–1.19)	0.98 (0.53–1.82)	1.13 (0.91–1.42)	1.0 (ref)	1.11 (0.63–1.97)	0.97 (0.55–1.73)

Values are n (range) unless otherwise indicated. Model 1: adjusted for matching factors (age, race [women only], month of blood draw, fasting status, and smoking status). Model 2: adjusted for matching factors, triglycerides, HDL-C, LDL-C, hsCRP, and HbA_{1c}. Model 3: adjusted for matching factors, triglycerides, HDL-C, LDL-C, hsCRP, HbA_{1c}, parental history of MI before age 60, pack-years of smoking, physical activity, hypertension, diabetes, hypercholesterolemia, BMI, aspirin use and post-menopausal hormone use (women only).

IC = immune complexes; other abbreviations as in Table 1.

physical activity, hypertension, diabetes, hypercholesterolemia, BMI, aspirin use, and post-menopausal hormone use (women only). In men, the improvement was smaller: 0.728 to 0.731 and 0.735 with the addition of OxPL/apoB and Lp(a), respectively. Comparing OxPL/apoB and Lp(a) with other standard biomarkers, the magnitude of association between a 1-SD increase in OxPL/apoB and Lp(a) and risk of PAD was similar to that for LDL cholesterol and high-density lipoprotein cholesterol in men (Fig. 4).

Table 6 C-Statistic Values for Area Under the Receiver-Operating Characteristic Curves

	Women	Men
Traditional risk factors	0.725	0.728
Traditional risk factors + OxPL/apoB	0.756	0.731
Traditional risk factors + Lp(a)	0.761	0.735
Traditional risk factors + triglycerides	0.727	0.741
Traditional risk factors + HDL-C	0.726	0.761
Traditional risk factors + LDL-C	0.727	0.734
Traditional risk factors, OxPL/apoB, Lp(a)	0.759	0.736
Traditional risk factors, OxPL/apoB, Lp(a), triglycerides	0.762	0.749
Traditional risk factors, OxPL/apoB, Lp(a), triglycerides, HDL-C	0.763	0.769
Traditional risk factors, OxPL/apoB, Lp(a), triglycerides, HDL-C, LDL-C	0.763	0.780

Traditional risk factors: matching factors (age, race [women only], month of blood draw, fasting status, and smoking status), parental history of MI before 60 years of age, pack-years of smoking, physical activity, hypertension, diabetes, hypercholesterolemia, BMI, aspirin use, and post-menopausal hormone use (women only).

Abbreviations as in Table 1.

Discussion

This study demonstrates that OxPL/apoB levels are positively associated with risk of PAD in men and women, with no appreciable attenuation after adjustment for conventional risk factors. Risk of developing PAD was approximately doubled among those in the highest compared with the lowest tertile. The Lp(a), the main lipoprotein carrier of OxPL, was similarly associated with risk of PAD. By contrast, we found no consistent relationship of indirect measures of oxidized lipoproteins such as autoantibodies to MDA-LDL and apoB-immune complexes with risk of PAD.

The role of OxPL and the role of the innate and adaptive immune system in mediating many pro-atherogenic processes is well-established in experimental models (10,11,23). However, clinical evidence is limited due to the absence of adequate diagnostic and therapeutic tools to measure atherosclerosis. In particular, biomarkers to predict PAD have been lacking (24). The OxPL/apoB is a validated biomarker that reflects anatomical coronary, carotid and femoral artery disease, atherosclerosis, and predicts 15-year risk of myocardial infarction and stroke (7) and allows re-classification of almost one-third of patients in the intermediate Framingham risk category to either lower- or high-risk categories (25).

In this study, we demonstrate the novel observation that elevated levels of OxPL/apoB are associated with PAD in both men and women in nested case-control studies within 2, well-validated epidemiological cohorts. The odds ratios were robust, averaging approximately 2.0/1-SD increase,

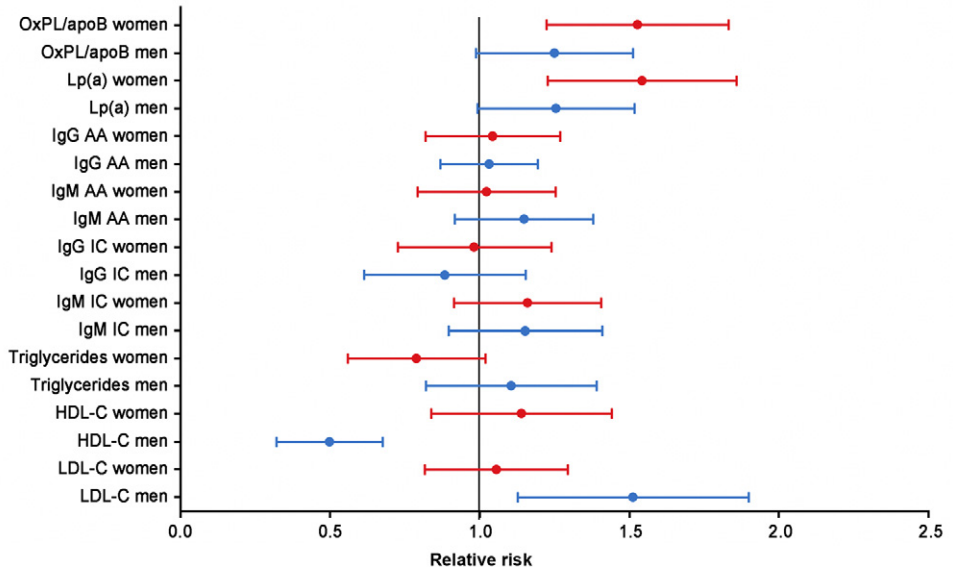

Figure 4 **Adjusted Relationship Between Oxidation-Specific and Standard Biomarkers and RR of PAD**

Adjusted for age, race (women only), fasting status, smoking, triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), high-sensitivity C-reactive protein, hemoglobin A_{1c} (HbA_{1c}), parental history of myocardial infarction before age 60, pack-years of smoking, physical activity, hypertension, diabetes, hypercholesterolemia, body mass index, aspirin use, and post-menopausal hormone use (women only).

AA = autoantibodies; IC = immune complexes; Ig = immunoglobulin; other abbreviations as in Figure 1.

which is in line with prior data on CVD endpoints (26,27). Interestingly, in women, the curve was shifted to the left compared with men, suggesting a stronger association with lower OxPL/apoB values. Furthermore, the OxPL/apoB relationship to PAD was different than the Lp(a) relationship in women, with a similar left shift. This might reflect differences in OxPL content among Lp(a) particles, with higher OxPL in Lp(a) associated with small isoforms (28). This hypothesis-generating observation needs to be replicated, but it does suggest OxPL/apoB might be particularly useful in women. Our finding that an easily measured marker of lipid oxidation is independently associated with risk of PAD also provides evidence that oxidative stress might indeed be an important contributor to atherosclerosis and provides a potentially useful tool to test this hypothesis in future studies.

We likewise found a positive association between Lp(a) and risk of PAD, an area where previous studies have been discordant. Some (29–36), but not all (30,37–39), cross-sectional studies tend to show positive associations between Lp(a) and PAD or PAD progression, and 1 longitudinal study found a positive association (36). In contrast to PAD, data on the role of Lp(a) in the development of CVD is fairly consistent for a modest, independent association (40).

A recent pooled analysis found that the RRs of CHD and ischemic stroke/1-SD increase in Lp(a) were 1.13 (95% CI: 1.09 to 1.18) and 1.10 (95% CI: 0.97 to 1.04), respectively, after adjusting for lipids and other conventional risk factors (41). We observed a pooled RR of PAD/1-SD that was more than twice as strong as the previous estimate for CHD (estimated ln[RR] of 0.12 vs. 0.31), suggesting that Lp(a) might be a stronger risk factor for PAD than for other forms of CVD; however, more studies are needed to confirm this observation definitively.

Despite a recent focus on OxPL and Lp(a) as cardiovascular disease risk factors, the etiologic link between Lp(a) and atherosclerosis remains unclear. Lp(a) mediates atherogenesis through mechanisms linked to its LDL and apo(a) components and its associated pro-inflammatory OxPL (42). A link was recently established between OxPL and Lp(a) in mediating macrophage apoptosis, a common mechanism for plaque progression and destabilization (43).

Given that atherosclerosis represents a dynamic process of lipid deposition and reverse transport, clearance of oxidized particles would be anticipated to prevent atherosclerotic progression. In this study, we measured autoantibodies to MDA-LDL, which would be anticipated to serve as a sink for circulating oxidized LDL particles. Some, but not all (44), previous studies found an inverse association between levels of IgM autoantibodies to MDA-LDL and cardiovascular disease, including coronary artery disease (45), hypertension (46), MI (47–49), and carotid and femoral atherosclerosis (14,15). It is interesting that we found an inverse relationship between IgG to MDA-LDL and risk of PAD in men but not in women. With this exception, we found no other consistent associations of either autoantibodies or IC to LDL with risk of PAD.

Strengths of our study include adjudicated cases of clinically meaningful PAD in both sexes, the high quality measurement of both biochemical and behavioral confounders, and the use of risk set sampling to select control subjects, which minimizes bias due to control subjects not accurately reflecting the source population from which the cases came. In addition, this is the first study to our knowledge that prospectively examines the association between novel cardiovascular risk factors OxPL/apoB, autoantibodies, and IC and risk of PAD.

Study limitations. Our study is not without limitation. There is a possibility of unmeasured or residual confounding, and because the NHS and HPFS studies contain predominantly white participants, we cannot necessarily generalize to minority populations, some of whom are at increased risk for PAD. However, we have no evidence that oxidative stress is less important in these populations, and indeed, recent evidence suggests that Lp(a) is strongly associated with CHD and ischemic stroke in both blacks and whites (50).

A potential limitation of studies of clinically significant PAD like this one is the possibility that some control subjects have undiagnosed PAD. Although this is a potential weakness of all studies of CVD events (e.g., individuals free of MI might nonetheless have asymptomatic coronary atherosclerosis), our results might be a conservative estimate of the association between OxPL/apoB and risk of PAD if control subjects have unrecognized PAD. However, all study participants are health professionals who report almost universal access to health care, which would tend to minimize undiagnosed cases. Regardless, our endpoint reflects clinically significant and meaningful cases of greatest concern to both patients and physicians. Finally, our results alone cannot definitively separate OxPL and Lp(a) as individual determinants of PAD, given their inherent biological interrelationship.

Conclusions

OxPL/apoB are positively associated with risk of PAD and are a unique oxidation-specific biomarker that it is not associated with other traditional CVD risk factors besides Lp(a), the major lipoprotein carrier of OxPL. Future research should continue to explore the mechanisms that link oxidation to risk of PAD and test whether modifiable risk factors, potentially including novel therapies that reduce levels of OxPL, might prevent the development of atherosclerotic diseases such as PAD.

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Key Words: autoantibodies ■ biomarker ■ immune complex ■ lipoprotein (a) ■ oxidized phospholipids ■ peripheral artery disease.

APPENDIX

For a supplementary table, please see the online version of this article.